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TWO CELL VOLUME REGULATORY SYSTEMS IN THE *LIMULUS* MYOCARDIUM: AN INTERACTION OF IONS AND QUATERNARY AMMONIUM COMPOUNDS

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Abstract

The horseshoe crab *Limulus polyphemus* is extremely euryhaline. Previous studies have shown it surviving in salinities ranging from 6% to 200% sea water. Blood osmotic concentration is hyperregulated in low salinities, but above 65% sea water *Limulus* is an osmoconformer. *Limulus* regulates cell volume when exposed to low salinity, despite a small intracellular free amino acid pool. Instead, the quaternary ammonium compound glycine betaine is the major nitrogenous osmotic solute in *Limulus* heart tissue. However, volume regulation is complete before intracellular glycine betaine concentrations change. Isolated heart tissue exposed to low salinity shows no change in glycine betaine levels in 24 h though volume regulation occurs. During the initial phase of volume regulation intracellular Na^+ and Cl^- content in the isolated tissue decreases markedly with exposure to low salinity. Therefore, *Limulus* utilizes two osmotic solute types during cell volume regulation: Na^+ and Cl^- initially and glycine betaine later.

Introduction

The utilization of free amino acids as intracellular osmotic solute to regulate cell volume in euryhaline invertebrates exposed to external osmotic stress has been considered ubiquitous (Gilles, 1979; Pierce and Amende, 1981). Free amino acids often make up more than 50% of the intracellular osmotic solute in marine invertebrates (Gilles, 1979). The levels of these intracellular free amino acids are adjusted in response to changes in extracellular osmotic concentration, controlling the volume of water in the cells. In contrast to other euryhaline invertebrates, the tissues of the horseshoe crab, *Limulus polyphemus*, contain only low levels of free amino acids, making up 10% or less of the osmotically active substances (Bricteux-Gregoire *et al.*, 1966; Prior and Pierce, 1981) despite its wide salinity tolerance. *Limulus* has been found living in salinities ranging from 7 to 30 ppt (McManus, 1969) and survived in experimental salinities of 3 to 64 ppt (Robertson, 1970). Furthermore, the free amino acid concentration in *Limulus* tissue drops only slightly with acclimation to low salinity (Prior and Pierce, 1981).

Although *Limulus* cells do not utilize amino acids as a main osmotic solute, the total non-protein nitrogen content of *Limulus* tissue is substantial and changes considerably with external salinity (Bricteux-Gregoire *et al.*, 1966; Robertson, 1970). The identity of this nitrogenous solute is unknown, but there are some obvious possibilities. In particular, quaternary ammonium compounds are common in in-

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Abbreviations: HPLC, high performance liquid chromatography; MOPS, morpholinopropanesulfonic acid; mosm, milliosmoles per kilogram water; TCA, trichloroacetic acid; TLC, thin layer chromatography.

vertebrate tissues (Welsh and Prock, 1958; Gasteiger *et al.*, 1960; Beers, 1967; Robertson, 1980) and, where it has been tested, these compounds vary with salinity (Bricteux-Gregoire *et al.*, 1962, 1964; Dall, 1971; Norton and de Rome, 1980) but usually constitute only a minor part of the nitrogenous osmotic solute pool. Quaternary ammonium compounds are present in *Limulus* (Ackermann and List, 1958) and might account for at least part of the unidentified pool of osmotic solute in *Limulus*, although Levy (1967) was unable to demonstrate such a relationship.

Another possible pool of intracellular osmotic solute is inorganic ions, although the evidence indicating the use of these substances by osmotically stressed invertebrate cells is limited. However, inorganic ions play a role in volume regulation in the few invertebrate species studied, both as an initial source of solute (Kevers *et al.*, 1981) and throughout the acclimation period (Freel, 1978; Willmer, 1978). K^+, Na^+, and Cl^- have all been implicated in some combination (Freel, 1978; Kevers *et al.*, 1979; Treherne, 1980).

Because of its extreme euryhalinity and small amino acid pool, we have investigated the possible role of these alternate solutes in the cell volume regulation of *Limulus* heart tissue. The results show that the quaternary ammonium compound glycine betaine is the major nitrogenous osmotic solute in heart tissue taken from crabs acclimated to low salinities. However, isolated heart tissue exposed to low salinity shows no decrease in glycine betaine or K^+ content in 24 h, but rather a large decrease in Na^+ and Cl^- content. Therefore, *Limulus* utilizes both organic compounds and inorganic ions to regulate cell volume during low salinity stress. A preliminary report of these findings has appeared previously (Warren and Pierce, 1981).

Materials and Methods

Limulus *acclimated to low salinity*

Limulus, obtained from The Marine Biological Laboratory, Woods Hole, MA, were acclimated to salinities ranging from 930 mosm to 55 mosm, at 14–18°C, for 2–3 weeks. Natural sea water, diluted appropriately with deionized water, was used. After the acclimation period, blood and tissue samples were taken to determine the low-salinity acclimated levels of blood osmotic concentration, tissue amino acids, total non-protein nitrogen and quaternary ammonium compounds, as indicated below.

Blood was withdrawn from the acclimated animals by insertion of a syringe into the pericardial sinus at the joint between the prosoma and the opisthosoma. The blood was centrifuged at $20{,}000 \times g$ to remove cells and clots, and the osmotic concentration of the supernatant determined using a freezing point depression osmometer (Precision Systems Osmette).

The heart was exposed by cutting away a dorsal section of the carapace. A small section of the cardiac tissue was removed, blotted, and weighed. The tissue was then lyophilized and reweighed to determine tissue hydration, as percent wet weight lost by lyophilization.

Intracellular amino acids were extracted from the dried tissue samples by homogenization in 40% ethanol, followed by boiling and centrifugation to remove protein. The supernatant was lyophilized and the residue resuspended in lithium citrate buffer (pH 2.2). The amino acid composition of this solution was determined with an amino acid analyzer (JOEL JLC-6AH).

Tissue non-protein nitrogen concentrations were also determined. Lyophilized heart tissue was homogenized in ice cold distilled water. Ice cold trichloroacetic acid

(TCA) was added to the homogenate to give a final concentration of 10% TCA. The precipitated protein was spun down at 20,000 × *g* and the supernatant frozen until analysis. A portion of each sample was added to tubes containing 0.1 g digestion mixture ("Sel-dahl" copper-selenite mixture, Scientific Products), followed by 0.5 ml of concentrated H_2SO_4. The tubes were heated to 320°C and the samples digested at that temperature for 2 h to break down nitrogenous compounds to ammonia (Lang, 1958). After cooling, the samples were diluted with distilled water and a portion from each was placed into a glass vial and neutralized with 50% KOH. The vials were quickly capped with rubber stoppers, each of which held a glass rod with a ground tip extending into the vial. Prior to insertion into the vial, a drop of 1 *N* H_2SO_4 was applied to the tip of each glass rod (Seligson and Seligson, 1951). The vials were rotated at an angle overnight. The ammonia released from the basic solution was trapped in the acid on the glass rods, forming ammonium sulfate. This was rinsed off with distilled water, and the ammonia content determined colorimetrically (Liddicoat *et al.*, 1975). Both ammonium sulfate and glycine betaine were run through the entire procedure as standards.

Intracellular quaternary ammonium compounds were measured in *Limulus* cardiac tissue by reineckate precipitation (Barnes and Blackstock, 1974). Tissue samples were extracted as described above for amino acid analysis, but the lyophilized supernatants were resuspended in distilled water rather than buffer. A portion of this solution from each tissue sample was applied to mixed bed ion-exchange columns (Dowex-1 and Amberlite-50, 2:1) and washed with water, removing any interferring amino acids. Then 1 *N* HCl was added and mixed with the washings, followed by saturated, filtered ammonium reineckate (pH 1). The quaternary ammonium compounds precipitated while standing overnight at 4°C. The precipitates were then filtered from each solution, using polycarbonate membrane filters (Bio-Rad, 0.2 μm) in syringe filter holders (Millipore). Once an entire sample was filtered, excess reineckate was removed by passing ether across the filter several times. The filter was removed, the precipitate dissolved in 70% acetone-water, and the absorbance read at 520 nm. Glycine betaine standards were run with each group of samples.

Preliminary investigations using standard paper and thin layer chromatography (TLC) (Bregoff *et al.*, 1953; Hayashi and Konosu, 1977) confirmed that the quaternary ammonium compounds glycine betaine and homarine were present in *Limulus* heart tissue. Concentrations of glycine betaine and homarine were then measured directly using high performance liquid chromatography (HPLC) (Altex). A reverse phase column (Spherisorb C-6, 5 μm particle size, Chromanetics) and a mobile phase of 0.1 *M* phosphate buffer (pH 3), containing 1 m*M* octane sulfonic acid as an ion-pairing reagent, were used to separate the compounds. Lyophilized samples were resuspended in water and a portion of each, appropriately diluted with mobile phase buffer, was injected onto the column. A UV monitor (Gilson) detected the compounds at 190 nm, and concentration was determined with a data processor (Shimadzu). Identification of peaks was verified by spiking samples with standards to obtain a single, larger peak and by collecting the column eluent containing each peak and running them on TLC.

Time course of acclimation

To determine the time course of events occurring during the low salinity acclimation process, changes in blood osmotic concentration, and cardiac tissue and blood glycine betaine levels were followed from the time of transfer of crabs to low

salinity to the end of the two-week acclimation period. Prior to the experimental period, animals were maintained at 10°C in artificial sea water (Instant Ocean, 930 mosm). Animals were then transferred from 930 mosm to 235 mosm; control animals were kept at 930 mosm.

Cardiac tissue samples were taken from both groups at various intervals throughout the two-week acclimation period. Glycine betaine in these tissues was measured as described above. Blood samples were also withdrawn from the animals in both groups as previously described. After centrifugation, the osmotic concentration of a portion of each blood sample was determined. The remainder of the blood samples were deproteinized by addition of appropriate amounts of ethanol to a final concentration of 40%, brought to a boil, and then centrifuged to remove the precipitate. The supernatant was analyzed for glycine betaine by HPLC.

Isolated tissue response to low salinity

The response of isolated *Limulus* hearts to low salinity stress was also investigated by measuring changes in tissue hydration, glycine betaine and intracellular ions. In order to demonstrate cell volume regulatory ability in isolated hearts, initial studies measured weight changes in isolated hearts exposed to low salinity. Hearts were dissected from *Limulus* acclimated to 930 mosm artificial sea water (10°C) and placed in saline (940 mosm, 10°C, ionic content in Table I). The hearts were carefully cleaned of any tissue debris and rinsed several times with fresh saline. Hearts were then put into either 940 mosm or 400 mosm saline and maintained at 10°C. (The 400 mosm saline is approximately the same osmotic concentration as blood taken from animals acclimated to 235 mosm.) The hearts were removed from the saline, blotted, and weighed at intervals up to 12 or 24 h. Changes in weight were expressed as percent initial wet weight for each heart.

To measure intracellular ions and glycine betaine, the dissected hearts were cleaned and split longitudinally along the ventral side and then cut into two sections across the width. One section of each heart was transferred to 400 mosm saline, while the other half was transferred to 940 mosm saline, both solutions containing ^{14}C-polyethylene glycol (MW 4000, New England Nuclear) as an extracellular marker (4 h required for complete equilibration in the extracellular space). The tissue pieces were maintained at 10°C with aeration and light shaking. Media and tissue were sampled at 6 and 12 h intervals. Each tissue sample at each interval was

TABLE I

Ionic concentrations of saline used in isolated Limulus *heart experiments.*

	m*M*
NaCl	420
$MgCl_2$	30
$MgSO_4$	20
KCl	11
$CaCl_2$	11
$NaHCO_3$	5
MOPS	5
mosm	940
pH	7.5

Lower salinities were made by dilution with distilled water, but maintaining the MOPS concentration.

divided into three pieces. Each piece was blotted and weighed. One was lyophilized and reweighed to determine tissue hydration, and then used for measurement of glycine betaine, as described previously. The ions were extracted from a second tissue piece with 1 *N* nitric acid. The third piece was solubilized in Protosol (New England Nuclear) and the ^{14}C-polyethylene glycol content measured by liquid scintillation counting. Radioactivity in a sample of the media was also determined, and ratios of these two counts were used to determine extracellular space.

Na^+ and K^+ concentrations were measured with an atomic absorption spectrophotometer (Perkin-Elmer Model 560). Samples of the tissue extracts and the incubation media were diluted with a solution containing 1% nitric acid and an excess of K^+ or Na^+ as applicable to prevent ionization. Standards also contained Ca^{++} and Mg^{++} in concentrations proportional to sea water. Chloride was measured in samples of extracting fluid and media using a chloridometer (Buchler-Cotlove).

Intracellular ion concentrations in acclimated animals

Intracellular ion concentrations were measured in hearts taken from animals acclimated to low salinity for 14 to 16 days. *Limulus* were acclimated to either 930 mosm or 235 mosm at 10°C. The acclimation salinity of 235 mosm results in blood osmotic concentrations of approximately 400 mosm, the osmotic concentration used for the isolated tissue experiments.

After the acclimation period, blood was collected from the animals and centrifuged as previously described. The hearts were then removed and quickly cleaned without rinsing in saline. Two pieces of tissue were excised from each heart and quickly processed: one was blotted, weighed, and lyophilized for determination of tissue hydration; the other was blotted, weighed, and placed in 1 *N* nitric acid for ion extraction. Na^+, Cl^-, and K^+ were measured as previously described. The remainder of each heart was incubated in blood, collected from the animal, to which ^{14}C-polyethylene glycol was added. The tissue sections were maintained with aeration and shaking for 4 h, to allow equilibration of the polyethylene glycol in the extracellular space. A piece of the heart was then removed, blotted, and weighed. The tissue and a portion of the blood were solubilized in Protosol and radioactivity determined by liquid scintillation counting. All intracellular ion concentrations were calculated with correction for the extracellular space (Freel *et al.*, 1973).

Statistical analysis

Statistical significance was determined by analysis of variance and Student's *t* test. A probability of $P < 0.02$ was considered significant. All data are expressed as means ± S.E.

RESULTS

Limulus *acclimated to low salinities*

In salinities from 700 mosm to 930 mosm, the blood osmotic concentration of *Limulus* varies directly with that of the external medium. Over this salinity range, the blood is slightly hyperosmotic to the medium (16 to 27 mosm) (Fig. 1). In the more dilute salinities, from 55 mosm to 600 mosm, the blood osmotic concentration is maintained well above that of the medium (52 to 307 mosm) as both Robertson (1970) and Mangum *et al.* (1976) reported.

Tissue hydration remained constant over the entire salinity range tested (Fig. 2). Analysis of variance revealed no significant differences in treatment means.

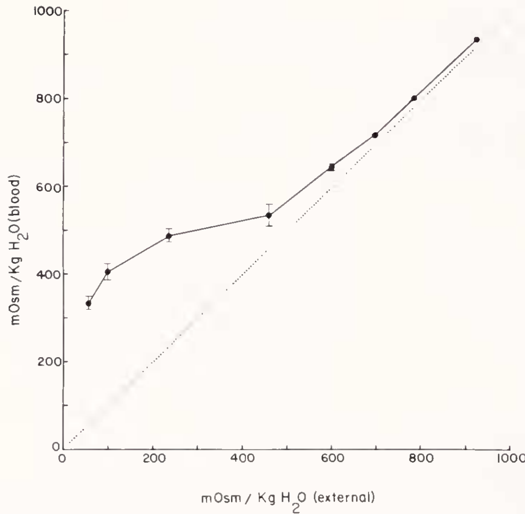

FIGURE 1. Blood osmotic concentration of *Limulus* acclimated to a range of salinities.

The total size of the free amino acid pool in heart tissue taken from *Limulus* acclimated to full strength sea water (930 mosm) is 170 μmoles/g dry weight. The pool size generally decreases with acclimation to lower salinities (Fig. 3). The major amino acid in *Limulus* heart tissue is taurine (Table II), making up nearly 50% of the total pool at 930 mosm and decreasing with salinity, especially at the lower acclimation salinities. In contrast, the total non-protein nitrogen in cardiac tissue is nearly 1300 μmoles/g dry weight and shows substantial decrease with acclimation to low salinity (Fig. 4).

A large portion of the non-protein nitrogen is accounted for by quaternary ammonium compounds, which are 750 μmoles/g dry weight in hearts of animals acclimated to 930 mosm (Fig. 4). Furthermore, the quaternary ammonium compound concentration decreases with acclimation to 700 mosm and 460 mosm salinities. Glycine betaine and homarine account for most of the quaternary ammonium compound pool (514 and 139 μmoles/g dry weight, respectively) at 930 mosm acclimation salinity (Fig. 5). Glycine betaine concentration decreases substantially over the range of acclimation salinities, whereas homarine shows only a slight decrease (Fig. 5).

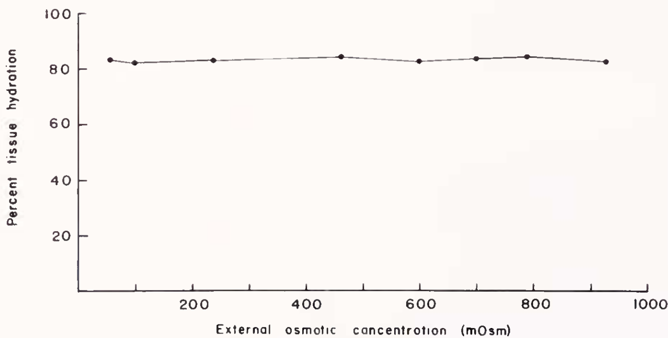

FIGURE 2. Percent hydration of heart tissue taken from *Limulus* acclimated to a range of salinities. Standard errors are smaller than the size of the points.

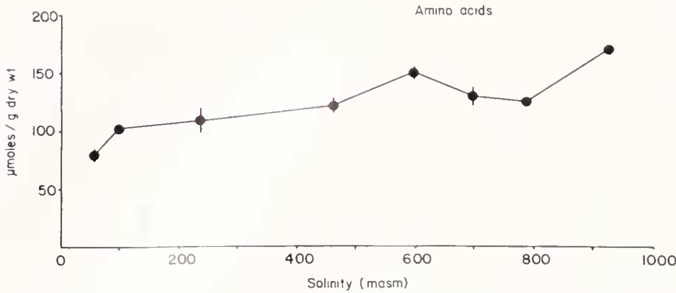

FIGURE 3. Total amino acid pool in heart tissue taken from *Limulus* acclimiated to a range of salinities.

Time course of acclimation

Following transfer directly from an external salinity of 930 mosm to 235 mosm, the blood osmotic concentration of *Limulus* drops rapidly to within 100 mosm of the final blood osmotic concentration during the first 24 h, and levels off within 48 h (Fig. 6 top). However, tissue glycine betaine does not decrease substantially until 48 h, and then only slowly declines up to day 7 (Fig. 6 middle). Blood glycine betaine concentrations reflect the tissue changes, not reaching a peak until 48 to 72 h and then gradually declining through day 14 (Fig. 6 bottom).

Isolated tissue response to low salinity

Isolated *Limulus* hearts taken from animals acclimated to 930 mosm and then exposed directly to 400 mosm saline gain 140% of initial wet weight in 2 h (Fig. 7). The weight then decreases to 123% of original by 12 h and finally recovers back to 117% of initial weight by 24 h.

In spite of this volume regulation, no significant change in tissue glycine betaine concentrations occurred after 6, 12, and 24 h of incubation in low salinity (Table III). However, significant decreases in inorganic ion concentrations occurred at these sampling intervals. Intracellular K^+ concentration decreases slightly in the tissues exposed to low salinity, but no more than can be accounted for by cell swelling (Table IV). The intracellular K^+ content (mmoles/kg dry weight) does not decrease in low salinity. Intracellular Na^+ and Cl^- concentrations decrease drastically during exposure of the isolated heart tissues to 400 mosm saline (Table V and VI). This decrease is significantly lower than that predicted by cellular hydration changes, and the Na^+ and Cl^- contents (mmoles/kg dry weight) also decrease significantly in these tissues exposed to low salinity.

TABLE II

Major amino acids (μmoles/g dry wt ± S.E.) in heart tissue taken from Limulus *acclimated to full-strength sea water (930 mosm).*

Tau	79.5 ± 5.7
Glu	24.9 ± 2.8
Pro	23.7 ± 3.3
Arg	21.7 ± 1.8
Ala	7.9 ± 1.1
Orn	4.8 ± 1.5
Asp	3.6 ± 0.8

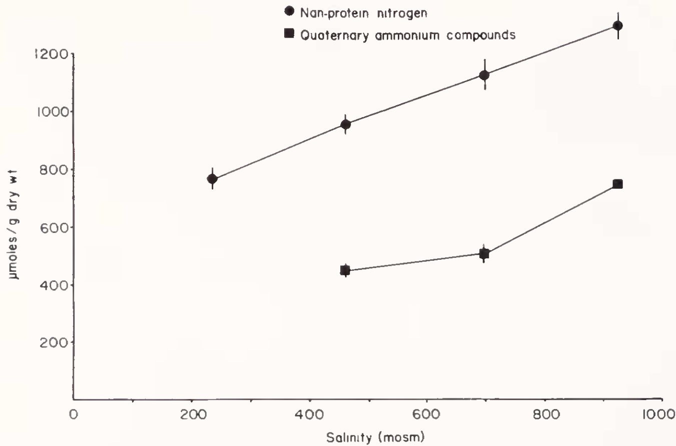

FIGURE 4. Non-protein nitrogen and quaternary ammonium compounds in heart tissue taken from *Limulus* acclimated to the salinities shown.

Ion concentrations in acclimated animals

The levels of intracellular inorganic ions in heart tissue taken from animals acclimated to low salinity are different from those in the isolated heart tissue following exposure to low salinity (Table VII). Intracellular Na^+ and Cl^- levels in the low salinity acclimated animals are significantly lower than in the high salinity acclimated animals. However, the Na^+ and Cl^- levels in the low salinity acclimated animals are significantly increased from the levels in the isolated tissue after a 12 h exposure to low salinity. Furthermore, intracellular K^+ in the low salinity acclimated animals is significantly decreased from the levels in high salinity acclimated animals, even though K^+ content in the isolated tissues did not decrease.

DISCUSSION

The extreme euryhalinity of *Limulus* can be accounted for by two general physiological processes. First, we found as did Robertson (1970) that *Limulus* is an

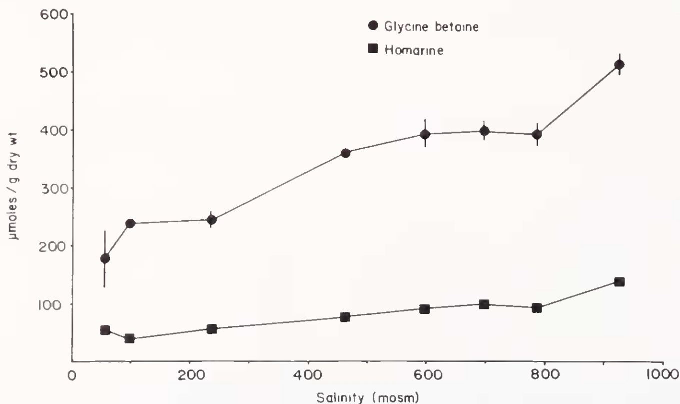

FIGURE 5. Glycine betaine and homarine concentrations, measured by HPLC, in heart tissue taken from *Limulus* acclimated to a range of salinities.

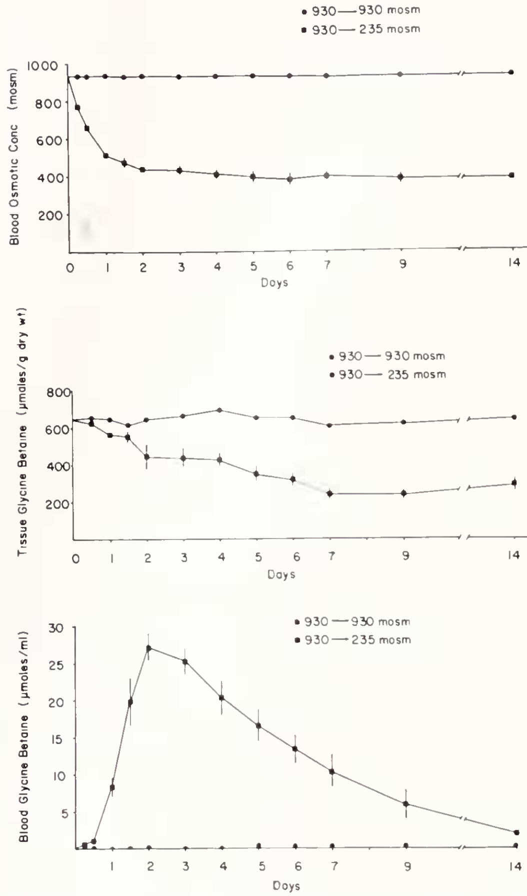

FIGURE 6. Time course of changes in blood osmotic concentration (top), tissue glycine betaine (middle) and blood glycine betaine (bottom) of *Limulus* acclimated to 930 mosm and exposed to 235 mosm. Values for control animals kept in 930 mosm are also shown.

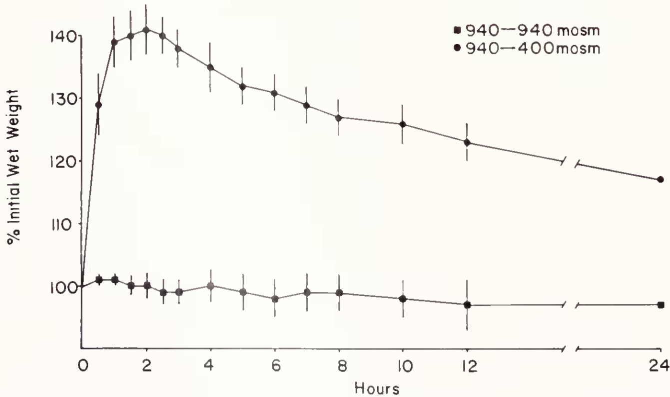

FIGURE 7. Time course of changes in wet weight, as % initial wet weight, of isolated hearts, taken from *Limulus* acclimated to 930 mosm, and exposed to 400 mosm or 940 mosm.

TABLE III

Glycine betaine (μmoles/g dry wt ± S.E.) in isolated heart tissue from Limulus *acclimated to 930 mosm.*

	940 mosm	400 mosm
6 h	599 ± 24	633 ± 15
12 h	621 ± 16	631 ± 27
24 h	585 ± 21	620 ± 21

The low salinity values are not significantly different from the high salinity controls.

osmoregulator in salinities below 600 mosm. Second, *Limulus* has a substantial ability to regulate cell volume. The basis of this cellular mechanism is the utilization of two types of intracellular osmotic solutes: small molecular weight nitrogenous compounds and inorganic ions. Unlike many invertebrates, *Limulus* has only a small intracellular free amino acid pool. Instead, the quaternary ammonium compound glycine betaine is the major nitrogenous osmotic solute in *Limulus* heart tissue. This compound is a common constituent in many invertebrates, but usually in small amounts (Robertson, 1961, 1965, 1980; Beers, 1967). Glycine betaine occurs in substantial amounts in some molluscs (*Mytilus*, Bricteux-Gregoire *et al.*, 1964; *Tapes*, Norton and de Rome, 1980), in association with substantial amino acid pools.

Free amino acid concentrations in the cells of intact euryhaline invertebrates normally fall rapidly during low salinity stress, often reaching the final lowered concentration within a day or two (Dall, 1975; Bartberger and Pierce, 1976). In contrast, glycine betaine concentrations slowly decreased over 7 days in the heart tissues of *Limulus* acclimating to low salinity, long after the drop in blood osmotic concentration occurred. However, the 100-fold increase of blood glycine betaine concentrations during the period of glycine betaine decrease in the tissues indicates that glycine betaine is effluxed intact from the cells, in a manner similar to free amino acid utilization by other species (Pierce and Amende, 1981). Thus, glycine betaine is only slowly utilized as osmotic solute and not at all in the initial stages of salinity acclimation in *Limulus* heart tissue. This is confirmed by our isolated tissue experiments.

The isolated *Limulus* heart volume regulates during exposure to hypoosmotic media. The pattern of volume regulation by this tissue is typical of that found in

TABLE IV

Intracellular K^+ in isolated heart tissue from Limulus *acclimated to 930 mosm.*

	mmoles/kg H_2O			mmoles/kg dry wt	
Salinity	940 mosm	400 mosm	Predicted*	940 mosm	400 mosm
6 h	112.6 ± 7.5	74.7 ± 2.9	75.0 ± 5.2	458 ± 29	432 ± 14
12 h	113.0 ± 5.7	83.2 ± 3.2	73.6 ± 4.3	432 ± 28	453 ± 11
24 h	114.5 ± 5.3	88.6 ± 3.5	79.3 ± 4.6	458 ± 20	511 ± 13

* Calculated according to Freel *et al.* (1973).

The data are expressed two ways. K^+ concentration (mmoles/kg H_2O) decreases during low salinity exposure but only as much as predicted by changes in tissue hydration. There is no significant decrease in K^+ content (mmoles/kg dry wt), indicating that K^+ is not used as osmotic solute.

TABLE V

Intracellular Na^+ in isolated heart tissue from Limulus *acclimated to 930 mosm.*

	mmoles/kg H_2O			mmoles/kg dry wt	
Salinity	940 mosm	400 mosm	Predicted*	940 mosm	400 mosm
6 h	237.7 ± 13.0	79.0 ± 16.9	153.1 ± 8.9	913 ± 51	437 ± 89
12 h	228.9 ± 17.6	46.3 ± 6.3	144.5 ± 10.6	905 ± 72	273 ± 34

* Calculated according to Freel *et al.* (1973).

Na^+ concentration (mmoles/kg H_2O) decreases substantially during low salinity exposure, significantly more than predicted by hydration changes. Na^+ content (mmoles/kg dry wt) also shows a very significant decrease during low salinity stress, indicating that Na^+ is used as osmotic solute.

other cell types: a rapid swelling followed by an incomplete recovery (reviewed by Gilles, 1979). Cellular volume regulation in response to hypoosmotic stress is achieved by a reduction in the amount of intracellular organic osmotic solute. In most invertebrate cells the solute reduction is accomplished by a rapid efflux of amino acids, but in the isolated *Limulus* heart, glycine betaine levels remained constant throughout the 24 h exposure to low salinity even though volume regulation was occurring. Thus, the initial control of cell volume in the *Limulus* tissue must rely on an alternate solute source. Our results indicate that intracellular Na^+ and Cl^- provide that function. Intracellular Na^+ and Cl^- contents decrease in isolated heart tissue exposed to low salinity, and the decrease occurs quickly, within the first 6 h. Therefore, the isolated heart volume regulates utilizing the high intracellular Na^+ and Cl^- contents as osmotic solute, without any changes in the level of glycine betaine.

The utilization of Na^+ and Cl^- as initial osmotic solute explains the lag time between the decline in blood osmotic concentration and changes in glycine betaine in the cells of the acclimating whole animal. Na^+ and Cl^- probably serve as the initial osmotic solute during the first day or two of exposure of the whole animal to low salinity, with the glycine betaine utilization occurring slowly as the first week of acclimation proceeds. In part, glycine betaine replaces Na^+ and Cl^- as osmotic solute during the acclimation process, shown by the partial return of Na^+ and Cl^- levels towards original.

TABLE VI

Intracellular Cl^- in isolated heart tissue from Limulus *acclimated to 930 mosm.*

	mmoles/kg H_2O			mmoles/kg dry wt	
Salinity	940 mosm	400 mosm	Predicted*	940 mosm	400 mosm
6 h	199.9 ± 16.0	60.3 ± 8.7	124.7 ± 9.4	780 ± 52	352 ± 56
12 h	195.8 ± 13.5	39.5 ± 4.6	135.8 ± 12.8	762 ± 48	221 ± 26
24 h	201.6 ± 15.3	38.8 ± 5.1	141.0 ± 8.3	834 ± 76	213 ± 24

* Calculated according to Freel *et al.* (1973).

Cl^- concentration (mmoles/kg H_2O) decreases substantially during low salinity exposure, significantly more than predicted by hydration changes. Cl^- content (mmoles/kg dry wt) also shows a very significant decrease during low salinity stress, indicating that Cl^-, like Na^+, is used as osmotic solute.

TABLE VII

Intracellular ion content (mmoles/kg dry wt ± S.E.) of heart tissue taken from Limulus *acclimated to 930 mosm or 235 mosm.*

	Acclimated animals		Isolated tissue*	
Salinity	940 mosm	235 mosm	940 mosm	400 mosm
Na^+	873 ± 47	401 ± 62	905 ± 72	273 ± 34
Cl^-	853 ± 57	347 ± 55	762 ± 48	221 ± 26
K^+	443 ± 16	361 ± 11	432 ± 28	453 ± 11

* Ion contents of isolated heart tissue after a 12 h exposure to 940 or 400 mosm are included for comparison.

Our results show that the utilization of glycine betaine as osmotic solute in *Limulus* heart tissue is very different from the mechanisms of free amino acid regulation in other euryhaline invertebrates. It is clear that *Limulus* cells utilize two very different types of osmotic solute. The solute control mechanisms are unknown and we are currently investigating them. However, our study indicates that the mechanisms controlling each of the solute levels are different, functioning with separate time courses. In spite of this difference, the mechanisms are coordinated so that cell volume is rapidly reduced by Na^+ and Cl^- efflux, and the later glycine betaine efflux continues the acclimation process, maintaining and perhaps finely adjusting cell volume. Thus, there seem to be two permeability control systems acting in concert to regulate cell volume.

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